PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

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Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
80472-5		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/CA99/00813	03/09/1999	03/09/1998
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant THE UNIVERSITY OF BRITISH COLUMBIA et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 6 sheets.

3. This report contains indications relating to the following items:

I	☒	Basis of the report
II	☐	Priority
III	☐	Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
IV	☒	Lack of unity of invention
V	☒	Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
VI	☐	Certain documents cited
VII	☒	Certain defects in the international application
VIII	☒	Certain observations on the international application

Date of submission of the demand	Date of completion of this report
07/02/2000	23.11.2000
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Herrero, M Telephone No. +49 89 2399 8542

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/CA99/00813

I. Basis of the report

1. This report has been drawn on the basis of *(substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments (Rules 70.16 and 70.17).)*:
Description, pages:

1-8,12-14,16-20	as originally filed			
9-11,15	as received on	06/09/2000	with letter of	29/08/2000

Claims, No.:

1-5,6 (part)	as originally filed			
6 (part),7-17	as received on	02/09/2000	with letter of	25/08/2000

Drawings, sheets:

1/3-3/3	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   ☐ the description, pages:
   ☐ the claims, Nos.:
   ☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:
   see separate sheet

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

   ☐ restricted the claims.

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☐ neither restricted nor paid additional fees.

2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

   ☐ complied with.

   ☒ not complied with for the following reasons:
   see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

   ☒ all parts.

   ☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-17
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-16
	No:	Claims	17
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations
see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

SECTION I

3. The amendments filed with the letter dated 25.08.00 introduce (apparently by mistake) subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following: the genomovar-specific primer pairs cited in the newly filed Claim 14, which are not supported by the corresponding disclosure on pages 7-9.

   Accordingly, Claim 14 as originally filed has been taken into account for the purposes of the present preliminary examination report.

4. Additional observations

   This preliminary examination report also takes into consideration pages 1/15 to 15/15 of the Sequence Listing (i.e. information concerning SEQ ID NOs 1 to 40).

SECTION IV

The subject-matter presently claimed can be clearly divided into the following separate inventions/groups of invention:

(1) Claims 1-16 (first invention): methods for the identification and speciation of bacteria belonging to the *Burkholderia cepacia* complex (Claims 1-7). Reagents (i.e. compositions comprising a suitable pair of polynucleotide primers according to Claims 8-10 and 13-14) and kits (Claims 11-12 and 15-16) for use in the methods of Claims 1-7.

(2) Claim 17 (second invention): vaccine composition for the treatment and prevention of infection with epidemic bacteria (belonging to genomovar III) of the *Burkholderia cepacia* complex comprising flagellin or a flagellin-derived antigen or a polynucleotide encoding flagellin or a flagellin-derived antigen.

It is *a priori* apparent that no single general inventive concept links the subject-mater of Claims 1-16 (first invention) to Claim 17 (second invention), contrary to the requirements of Rule 13.1 PCT.

Moreover, there is also no technical relationship among the methods, reagents and kits encompassed by Claims 1-16 and the vaccine composition of Claim 17 involving one or more of the same or corresponding special technical features that define a contribution which each of the claimed inventions, considered as a whole, makes over the prior art and therefore the requirements of Rule 13(2) PCT are not complied with.

SECTION V

2. CITATIONS AND EXPLANATIONS

2.1 The following document has been considered for the purposes of this report:

D1: Vandamme, P. et al (1997) Int. J. Syst. Bacteriol. **47**:1188-1200 (also cited in the application).

As mentioned on page 1 of the present application, it has been shown in D1 that the Gram negative bacterium *Burkholderia cepacia* actually consists of five different genomovars (or new species). Two of these genomovars have been given new species names, namely *Burkholderia multivorans* (formerly genomovar II) and *Burkholderia vietnamensis* (formerly genomovar V). As a collective, these two species together with *Burkholderia cepacia* genomovar I, *Burkholderia cepacia* genomovar III and *Burkholderia cepacia* genomovar IV have been designated as the *B. cepacia* complex.

2.2 The present application discloses a seemingly rapid procedure suitable for the identification and speciation of bacteria belonging to the *B. cepacia* complex present in a sample, which relies on the information provided by a *rec*A gene-based PCR assay. The application shows, in particular, that the described

process of speciation of *B. cepacia* bacteria based upon RFLP (restriction fragment length polymorphism) analysis of PCR-amplified *rec*A gene is highly discriminatory and demonstrates that the approach of the invention will clearly distinguish between all five of the current genomovars and also newly defined groups within the *B. cepacia* complex, i.e. the two sub-groups designated as RG-A and RG-B within genomovar III (cf page 6, lines 18-25).

(1) First invention: Claims 1-16

In the light of the available prior art the hereby claimed methods for the identification and speciation of bacteria belonging to the *Burkholderia cepacia* complex (Claims 1-7), compositions comprising pairs of primers for amplification (Claims 8-10 and 13-14) and speciation kits (Claims 11-12 and 15-16) would appear to relate to novel and inventive subject-matter susceptible of industrial applicability and therefore to satisfy the criteria set forth in Art. 33(2)(3) and (4) PCT.

(2) Second invention: Claim 17

Regarding the intended vaccine composition according to Claim 17 it is emphasized that no experimental support is found elsewhere in the application as originally filed on the basis of which it could be established that the expected technical effects, e.g. prevention of infection, are indeed obtained and thus is not possible to assess that the claimed subject-matter involves an inventive step (Art. 33(3) PCT).

The arguments put forward by the Applicants have been considered, however, the present Examining Authority is still of the opinion that the explanations given in the supporting description aimed at substantiating that the encoded protein (the product of the flagellin gene of *Burkholderia cepacia* genomovar III strains) is suitable for use as an antigen for development of vaccines against the most problematic strains in patients with cystic fibrosis (CF), are merely of a predictive and speculative nature (see in particular page 11, lines 9-11 and 19-21). Conversely, no information is available substantiating, for instance, the protection

of animals immunized with a composition comprising the flagellin antigen of interest against challenge with an infective strain belonging to the epidemic *Burkholderia cepacia* genomovar III.

SECTION VII

The expression "incorporated herein by reference" in respect of prior art documents on page 12, line 22 leads to a doubt as to whether the requirements of the description being self-contained are satisfied (see PCT Guidelines C-II, 4-17).

SECTION VIII

In the light of the supporting description (see page 4, lines 21-31 bridging over page 5, lines 1-11) it is evident that the composition comprising the pair of primers identified in present dependent Claim 9 is useful to carry out a non-specific PCR amplification of the *rec*A gene, i.e. said pair of primers is effective to amplify substantially the entire *rec*A gene from all known members of the *B. cepacia* complex.

In view of the foregoing, the intended composition according to dependent Claim 9 cannot be regarded as a composition suitable for the production of a diagnostic amplicon from the *rec*A gene as defined in independent Claim 8. The subject-matter encompassed by Claim 9 is therefore inconsistent with the definition of the composition of the invention according to Claim 8, contrary to Art. 6 PCT.

This deficiency (Art. 6 PCT) affects *mutatis mutandis* the subject-matter of appended Claims 11 and 12, insofar as these claims rely in part on the pair of primers in accordance to present Claim 9.

5' - TCG AGA CGC ACC GAC GAG - 3' SEQ ID No. 33

PCR product expected from *B. cepacia* strains of genomovar III = 378 bp.

Additional sequencing of the complete *recA* gene from *B. cepacia* complex strains M36, M54, Ral-3 and *B. pyrrocinia* LMG 14191^T or partial sequence analysis of PCR amplicons derived from strains ATCC 29464, ATCC 53617, ATCC 39277, ATCC 49709 and ATCC 53266 was performed. The phylogenies determined using partial sequencing of this type were identical to those determined using the full sequences (Fig. 2), however, two additional clusters, denominated as RG-C and RG-D were identified. Cluster RG-C was a novel group consisting of biocontrol strains Ral-3, ATCC 53266 and M54. Comparative alignment of the *recA* sequence from Ral-3 and M54 with all other complete *B. cepacia* sequences enabled the design of RG-C specific primers with the following sequences:

Forward Primer:

GTCGGGTAAAACCACGTG SEQ ID No. 39

Reverse Primer:

TCCGCAGCCGCACCTTCA SEQ ID No. 40

B. cepacia biocontrol strains BC-B, BC-F and AMMD all tested positive with this RG-C primer set. Thus, these primers can be used in analytical schemes for the presence of such primers, and also could be used for screening isolates for biocontrol properties.

A second novel *recA* group, RG-D, was identified which includes *B. pyrrocinia* LMG 14191^T and ATCC 32977, a strain of *B. cepacia* which produces the antibiotic xylocladin. This group is also shown in Fig. 2.

In addition to a method for identification and speciation of bacteria of the *B. cepacia* complex, the invention also provides reagents and kits suitable for carrying out this method. The reagents are generally polynucleotide primers or probes which bind to the *recA* gene of one or more strains of bacteria of the *B. cepacia* complex. One subset of the reagents of the invention are non-specific primers, such as used in Example 4 below, which are complementary to conserved regions found identically in strains of bacteria of the *B. cepacia* complex for which the sequences are given. A second subset of reagents in

accordance with the invention are primers/probes which can be used to selectively amplify and/or detect one genomovar of bacteria of the *B. cepacia* complex. The reagents of the invention may have a detectable or capturable label, for example a radioactive or fluorescent label or biotin, incorporated therein to facilitate evaluation of nucleotide sequence information.

Either of these types of primers/probes may be packaged in a kit with suitable reagents. These reagents may include discriminatory restriction enzymes, which are capable of producing distinctive fragment patterns to permit speciation of a bacteria-containing sample, or reagents suitable for PCR, nucleic acid sequencing and the like.

Once the species of a sample bacterium of the *B. cepacia* complex is determined using the method of the invention, it may be desirable (particularly where the bacteria is a member of an epidemic strain) to be able to provide a therapeutic agent which is effective in treating or preventing infection. Thus, the present invention further provides a vaccine composition based upon the antigenic properties of the flagellin of epidemic strains of *B. cepacia* complex for use in treating infections caused by certain species of the *B. cepacia* complex.

The use of flagellins as an antigen for vaccine purposes has been proposed in a variety of instances because of their location on the outside of bacterial cells. In the case of *B. cepacia* complex, however, Hales et al., *J. Bacteriol.* 180: 1110-1118 (1998), have reported that the flagellin gene (fliC) is "highly variable" and suggest its utilization as a biomarker for epidemiological and phylogenetic studies of *Burkholderia cepacia*. Such variability is inconsistent with the normal requirements that a vaccine antigen be highly conserved, such that its will be generally effective against variants of the target species. Thus, it was quite surprising to find that the subset of *B. cepacia* complex which is most transmissible have highly conserved flagellin genes which is suitable for use as a vaccine.

A total of 30 strains of bacteria of the *B. cepacia* complex were classified using the speciation method of the invention into groups based on the sequence of the *recA* gene, and were in addition characterized with respect to the BCESM (Burkholderia cepacia Epidemic Strain Marker) and *cblA* markers for highly transmissible strains of *B. cepacia*. As reflected in Table 1, a substantial portion of the genomovar III strains which were positive for one or both of these markers produced a

single RFLP pattern (Fig. 3, pattern G) after treatment with the restriction endonuclease *HaeIII.*

Exemplary sequences and a consensus sequence for the *B. cepacia* flagellin gene, which encodes the major subunit protein of the bacterial flagellum of *B. cepacia*, have been described in the literature by Hales et al. (supra). Using the same primers described by Hales, it has been determined that the flagellin genes of *B. cepacia* strains of *recA* type III-G (genomovar III, with *recA* RFLP pattern G) are highly conserved and do not vary considerably in DNA sequence. This indicates that the protein is also highly conserved in its structure and sequence, and thus is suitable for use as an antigen for development of vaccines against the most problematic strains in patients with cystic fibrosis (CF).

In contrast, *B. multivorans* strains of recA type F (genomovar II), which appear less problematic in patients with CF and do not generally spread among patients, have flagellin genes which are highly variable in sequence. These data suggest that with *B. multivorans* strains, a vaccine based on the flagellum may not protect against infection with all strains types as has been the case with the bacterium *Pseudomonas aeruginosa* in CF. Thus, the observation, that the flagellin gene is actually highly conserved in the most devastating epidemic *B. cepacia* strains infecting patients with CF is apparently unique to this subset of the species of the *B. cepacia* complex.

The observation that the flagellin gene is conserved among *B. cepacia* strains which are epidemic amongst patients with CF permits the development of a vaccine based on the encoded protein antigen. The vaccine can be prepared in a variety of ways. First, protein can be purified from bacterial strains representative of this group to obtain a purified antigen. Methods for purification of flagellin from bacteria are known in the art, and can be applied to recovery of purified flagellin from epidemic strains of *B. cepacia.* Purified antigen is then used as a vaccine, with or without an adjuvant. Vaccines of this type are generally administered by subcutaneous or intramuscular injection, although other routes of administration may also be suitable. Therapeutically effective levels and frequency of vaccine administration are determined by routine monitoring of antibody titers.

In addition to the use of purified flagellin isolated directly from bacteria, it will be appreciated that the same protein, or an immunologically effective portion thereof may

epidemic amongst patients with CF (Mahenthiralingam et al., *J. Clin. Microbiol.* 34: 2914-2920 (1996) and encode the BCESM (Mahenthiralingam et al., *J. Clin. Microbiol* 35: 808-816 (1997). The separate classification of these three strains based on the recA suggest that they may constitute a new species/genomovar group within the *B. cepacia* complex.

EXAMPLE 4

To obtain nucleotide sequence information about the *recA* genes of additional strains of bacteria of the *B. cepacia* complex (Table 1), samples of each strain were amplified using the following primers:

Forward Primer (BCR1)

TGACCGCCGAGAAGAGCAA SEQ ID No. 3

Reverse Primer (BCR 2)

CTCTTCTTCGTCCATCGCCTC SEQ ID No. 4

using a standard polymerase chain reaction mixture of 25 microlitres in volume (described in Mahenthiralingam et al., *J. Clin. Microbiol.* 35: 808-816 (1997)) containing 1.5 mM $MgCl_2$ and 10-20 ng of *B. cepacia* DNA. Amplification was performed as follows: 30 cycles of 1 min. at 94°C, 1 min. at 56°C, and 2 min. at 72°C, follow by a final 6 min. cycle at 72°C. This resulted in the amplification of a 1 kb DNA band corresponding to the *recA* gene of the *B. cepacia* strain being tested.

Several restriction enzyme were screened for their ability to reveal DNA sequence variation in this amplified gene which would be suitable for speciation of *B. cepacia*. The enzymes *Hae* III and *Alu* I were found to be suitably discriminatory. The restriction fragments produced by the enzyme *Hae* III were separated by agarose gel-electrophoresis, and the detected restriction fragment length polymorphisms (RFLPs) demonstrated that genomovar specific RFLPs could be generated using this approach. Representative patterns are shown in Fig. 3. (Bv = *B. vietnamiensis*, or genomovar V; Gv I = genomovar I; Bm = *B. multivorans* or genomovar II; Gv III = genomovar III and Gv IV = genomovar IV). This same approach has been applied to a panel of strains which are representative of all five genomovars of *B. cepacia* and been found to be able to distinguish strains of each genomovar (Table 1). This technique has also been applied to additional strains, and been

CTCTTCTTCGTCCATCGCCTC. SEQ ID No. 4

7. The method of claim 5, wherein the PCR amplification is carried out using the following primers:

Forward Primer

TGCGGATGGGCGACGGCG SEQ ID No. 20

Reverse Primer

CAGTTCTGTCGCTTGATCG. SEQ ID No. 21

8. A composition comprising a pair of polynucleotide primers for production of a diagnostic amplicon from the recA gene of bacteria that is a member of the *Burkholderia cepacia* complex, said pair of primers hybridizing with each of the polynucleotides whose sequences are given by Seq. ID. Nos. 1, 2 and 5-19 to produce as an amplification product a diagnostic amplicon which can provide diagnostic information concerning the member of Burkholderia cepacia complex.

9. The composition of claim 8, wherein the polynucleotide primers have the sequences:

Forward Primer

TGACCGCCGAGAAGAGCAA SEQ ID No. 3

Reverse Primer

CTCTTCTTCGTCCATCGCCTC. SEQ ID No. 4

10. The composition of claim 8, wherein the polynucleotide primers have the sequence:

Forward Primer

TGCGGATGGGCGACGGCG SEQ ID No. 20

Reverse Primer

CAGTTCTGTCGCTTGATCG. SEQ ID No. 21

11. A kit for speciation of bacteria of the *Burkholderia cepacia* complex, comprising, in packaged combination, a pair of polynucleotide primers in accordance with any of claims 8 - 10, and a discriminatory restriction endonuclease.

12. The kit of claim 11, wherein the restriction endonuclease is *HaeIII* or *AluI*.

13. A composition comprising a genomovar-specific primer pair effective under stringent PCR conditions to produce amplification products by amplification of at least a portion of the *recA* gene of bacteria belonging to one genomovar of the *B. cepacia* complex, but not to produce amplification products from bacteria belonging to other genomovars.

14. The composition according to claim 13, wherein the genomovar-specific primer pairs are selected from among the following primer pairs given by Seq ID Nos.: 23 and 24, 25 and 26, 27 and 28, 29 and 30, 31 and 32, or 33 and 34.

15. A kit for speciation of bacteria of the *Burkholderia cepacia* complex, comprising, in packaged combination, a pair of genomovar-specific polynucleotide primers in accordance with claims 13 or 14 and a discriminatory restriction endonuclease.

16. The kit of claim 15, wherein the restriction endonuclease is *HaeIII* or *AluI*.

17. A vaccine composition for treatment and prevention of infection with bacteria of the *Burkholderia cepacia* complex, wherein the bacteria is a member of genomovar III and has a nucleotide sequence for the recA gene which produces a G-type RFLP pattern when analyzed with the restriction enzyme HaeIII, and wherein the vaccine composition comprises flagellin or a flagellin-derived antigen or a polynucleotide encoding flagellin or a flagellin-derived antigen, said flagellin or flagellin-derived antigen being obtained from the bacteria that is a member of genomovar III and that has a nucleotide sequence for the recA gene which produces a G-type RFLP pattern when analyzed with the restriction enzyme HaeIII.